Simvastatin pretreatment reduces the severity of limb ischemia in an experimental diabetes model

Cüneyt Köksoy, MD,[a] Erpulat Oziş, MD,[a] Atıl Çakmak, MD,[a] Uğur Yazgan, PhD,[b] Aylin Okcu-Heper, MD,[c] Aslıhan Köksoy, MD, PhD,[d] Ediz Demirpençe, MD,[e] and **U. Deniz Dinçer, MD, PhD,[d]** *Ankara, Turkey*

Objective: **The purpose of this study was to examine the effects of simvastatin pretreatment in the setting of acute limb ischemia–reperfusion injury in an experimental diabetes model that is associated with a high risk for limb loss.**
Methods: **Adult male Sprague-Dawley rats were randomized into two groups. Diabetes was induced in the first group by intravenous streptozotocin injection. The second group served as the nondiabetic group. Eight weeks after the streptozotocin injection, half of the rats in the diabetic and the nondiabetic groups were further randomized to receive either intraperitoneal simvastatin (1 mg/kg per day) or saline treatment for 6 weeks. Bilateral hind-limb ischemia was induced for 4 hours by the tourniquet method. After 24 hours of reperfusion, tissue samples were collected from the gastrocnemius and anterior tibial muscles bilaterally for measurement of muscle edema, percentage of necrosis, and malondialdehyde (MDA), glutathione, and myeloperoxidase (MPO) levels.**
Results: **Ischemic injury was more prominent in diabetic animals. The diabetic animals with limb ischemia exhibited a 7% increase in tissue edema, a 47% increase in muscle necrosis and MPO level, and a 15% reduction in glutathione levels compared with the nondiabetic animals ($P < .05$). Simvastatin treatment with 1 mg/kg for 6 weeks reduced the ischemic injury. Simvastatin pretreatment led to a 71% reduction in muscle necrosis in diabetic animals ($P < .001$). The protective effects of simvastatin pretreatment also correlated with a 23% improvement in tissue edema, a 75% reduction in tissue myeloperoxidase content, and a 71% increase in glutathione levels in diabetic animals ($P < .01$). Furthermore, skeletal muscle injury, characterized by tissue edema and leucosequestration, was significantly less severe with simvastatin pretreatment compared with the nondiabetic animals ($P < .01$).**
Conclusion: **Simvastatin pretreatment reduced limb ischemia–reperfusion injury in diabetic and nondiabetic animals. We conclude that simvastatin pretreatment may be a potential therapeutic intervention for skeletal muscle ischemia–reperfusion injury in the clinical setting. (J Vasc Surg 2007;45:590-6.)**

Clinical Relevance: **In this study, we obtained results suggesting that pretreatment with simvastatin for 6 weeks ameliorated the tourniquet-induced skeletal muscle ischemia–reperfusion injury in diabetic and nondiabetic rats. Our results hint that pretreatment with 3-hydroxy-3 methyl-glutaryl-coenzyme A reductase inhibitors (statins) may be useful in preventing and reducing the severity of acute ischemic events. These effects may be most prominent in patients suffering from circulation challenges such as those seen in diabetic patients. Nevertheless, this issue requires clinical evidence.**

Diabetes mellitus (DM) is a major risk factor for cardiovascular morbidity and mortality.[1] Peripheral arterial disease (PAD) is one of the major complications of DM.[2] DM increases the incidence and severity of limb ischemia approximately twofold to fourfold.[3] Diabetic PAD often affects distal limb vessels, limiting the potential for collateral vessel development and reducing the options for revascularization. Critical limb ischemia requiring amputation develops more frequently in patients with DM than it does in others.[4] For patients aged 65 to 74 years, DM heightens the risk of amputation more than 20-fold, putting these patients at great risk for limb loss.[4]

Mortality risk is also particularly increased in diabetic patients with PAD.[1,2] Patients with PAD have a greatly elevated risk of myocardial infarction and stroke, and are six times more likely to die from cardiovascular causes than those without the disease.[5]

Treatment strategies for intensive blood-glucose control substantially decrease the risk of microvascular complications such as retinopathy and nephropathy, but not macrovascular disease, including PAD, coronary artery disease, and stroke in patients with type 2 diabetes.[6] None of the individual drugs had a substantial effect on cardiovascular outcomes.[6] Other strategies rather than glucose control are therefore necessary to reduce the risk of cardiovascular complications in DM.

The 3-hydroxy-3-methyl-glutaryl CoA reductase inhibitors (statins) are found to reduce the incidence of stroke, myocardial infarction, and mortality from vascular complications in patients with vascular disease or vascular risk factors.[7-12] Statins have antioxidant, antithrombotic,

From the Departments of General Surgery,[a] Pathology,[c] and Biophysics,[d] Faculty of Medicine, and Department of Pharmacology, Faculty of Pharmacy,[b] Ankara University; and the Department of Biochemistry, Faculty of Medicine, Hacettepe University.[e]
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Correspondence: Cüneyt Köksoy, MD, Ibni Sina Hastanesi K4, Samanpazari 06100 Ankara, Turkey (e-mail: cuneytkoksoy@hotmail.com).
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anti-inflammatory, fibrinolytic, and angiogenic effects. Statins also increase nitric oxide production and block the interaction between leukocytes and the endothelium. Recent clinical[7-11,13] and experimental[14-18] evidence suggests that some beneficial effects of statins are independent of their lipid-lowering properties and are due to their so-called pleiotropic effects.[19] Furthermore, several clinical studies have shown beneficial effects of statins in chronic limb ischemia. Statin use improves walking distance in patients with chronic limb ischemia[20] and is also associated with improved graft patency and limb salvage after infrainguinal bypass grafting.[21,22] Little is known about the effects of statins on acute limb ischemia, however.

We hypothesized that statins could also reduce peripheral ischemia–reperfusion injury because of their pleiotropic effects. To test this hypothesis, we investigated the effects of simvastatin pretreatment on the severity of acute limb ischemia–reperfusion injury in an experimental diabetes model that is associated with a high risk for limb loss.

MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 200 to 250 grams were housed under diurnal lighting conditions (12 hours of darkness and 12 hours of light). Before the experiment, they were made to fast overnight but were given free access to water. Animal housing, care, and application of experimental procedures were all done in accordance with the *Guidelines for Care and Use of Laboratory Animals,* published by the National Society for Medical Research and the National Institutes of Health. All of the animal experiments described were approved by the Ethics Committee of the Faculty of Medicine, Ankara University.

Experimental diabetes model. Rats were randomly divided into two groups. Diabetes was induced in the first group by 45-mg/kg streptozotocin (STZ) (streptozocin Zanosar, Pharmacia & Upjohn, Kalamazoo, Mich) in 0.9% physiologic serum, administered intravenously through the tail vein under ether anesthesia. Three days after the STZ injection, blood glucose levels were measured, and the animals with glucose levels >300 mg/dL were enrolled as diabetics. Only rats with blood glucose levels >300 mg/dL were entered into the experimental protocols. Animals receiving STZ without glucose levels >300 were excluded from the study. The second group served as the nondiabetic group and received saline alone.

Simvastatin pretreatment. Eight weeks after the STZ injection, both diabetic and nondiabetic groups were randomly divided into two subgroups and either 1 mg/kg per day of simvastatin (Merck & Co, Inc, Rahway, NJ) or an equivalent volume of saline was administered intraperitoneally for 6 weeks.[23] For the preparation of simvastatin, 4.0 mg was dissolved in 0.1 mL 95% ethanol and 0.15 mL 0.1 N sodium hydroxide was added and heated for 2 hours at 500°C. This was neutralized with hydrogen chloride, and when a pH of 7.2 was reached, distilled water was added, after which the total dilution volume was 1 mL. The 4.0 mg/mL stock solution was preserved at –20°C for further use. A total of 36 rats were studied in four groups: (1) 10 nondiabetic, (2) 7 nondiabetic pretreated with statin, (3) 10 diabetic, and (4) 9 diabetic pretreated with statin.

Hind-limb ischemia model. All the rats in each group underwent 4 hours of lower extremity ischemia and 24 hours of reperfusion. Rats were anesthetized with ketamine (50 mg/kg, intraperitoneally) (Ketalar, Parke-Davis, Morris Plains, NJ) and xylazine (10 mg/kg, intraperitoneally) (Rompun, Bayer, Leverkusen, Germany). Occlusion of the lower extremity was performed by the tourniquet method, as described previously.[24] The tourniquet (rubber band, No. 15, MAS, Istanbul, Turkey) was looped six times briefly as proximally as possible on the thigh. After 4 hours of ischemia, reperfusion was initiated by releasing the tourniquet. The rats were hydrated by an intraperitoneal injection of 40-mg/kg normal saline every 2 hours for 8 hours and allowed free access to water.

Biochemical analysis and tissue sampling. After 24 hours of reperfusion, all rats were anesthetized with ketamine (200 mg/kg, intraperitoneally), and after laparotomy, 5-mL blood samples were obtained from the inferior vena cava and preserved at –20°C for further use. Plasma glucose concentrations, glycosylated hemoglobin (HbA_{1c}) levels, and serum levels of cholesterol, triglyceride, lipoproteins (very-low-density lipoprotein [VLDL], high density lipoprotein [HDL]), and creatinine phosphokinase were analyzed for possible effects of simvastatin therapy and DM.

Tissue samples were obtained from the right gastrocnemius (whole muscle) to measure of muscle edema. The left gastrocnemius was used for measurement of percentage of necrosis. Bilaterally, whole anterior tibial muscles were used for measurement of malondialdehyde (MDA), glutathione, and myeloperoxidase (MPO) levels. Samples were obtained randomly.

Determination of tissue edema. The extent of skeletal muscle edema was determined by determining the ratio of the wet to dry tissue weight of the gastrocnemius sections.[25] The muscle samples were weighed immediately for measurement of the wet weight and then freeze-dried for 48 hours with a freeze-dry system (Heto Drywinner, Thermo Life Sciences, Basingstoke, UK) for measurement of the dry weight. The tissue water content was expressed as water content (%) = [(wet weight – dry weight)/wet weight] × 100.

Determination of muscle necrosis. Muscle necrosis was measured with nitroblue tetrazolium dye to stain the viable muscle.[26] The harvested muscles were immediately divided into three equal slices and weighed on a scale. The slices were incubated in 0.05% nitroblue tetrazolium solution for 20 minutes in a dark room. Viable muscle stained deep blue, whereas nonviable muscle remained pale and unstained (Fig 1).

The outline of the muscle slices and the areas of necrosis were outlined on a transparency. The total surface area and the area of necrosis were calculated for each slice by the use of computerized planimetry. The amount of muscle necrosis for each slice was measured by the ratio of area of necrosis/total surface area of the slice multiplied by the weight of the slice. The total sum of the necrotic

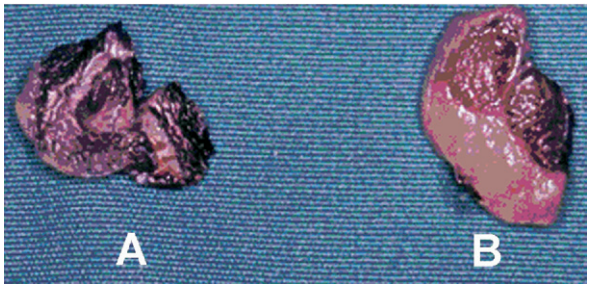

Fig 1. The appearance of gastrocnemius muscles stained with nitroblue tetrazolium dye just before planimetry. **A,** Viable muscle stained dark, and **(B)** nonviable muscle remained pale and unstained.

tissue of each muscle slice was determined by the amount of necrosis in each muscle and was expressed as a percentage. Percentage of necrosis = (total unstained area/total area) × 100.

Measurement of malondialdehyde. MDA is the end product of the major chain reactions leading to oxidation of polyunsaturated fatty acids, and measurement of MDA content is the most widely used method for assessing lipid peroxidation.[27] Tissues were homogenized in ice cold Tris/potassium chloride buffer (50 mmol, pH 7.4) using a homogenizer. Tissue MDA levels were determined according to the Uchiyama and Mihara method.[28] Tissue MDA level was determined by a method based on the reaction with thiobarbituric acid (TBA) at 90° to 100°C. In the TBA test reaction, MDA and TBA react together for production of a pink pigment with absorption maximum at 532 nm. The results were expressed as nanomoles per gram of wet muscle tissue weight.

Measurement of myeloperoxidase activity. Neutrophil infiltration after reperfusion injury was assessed by the increase in tissue MPO enzymatic activity.[29] Tissue samples were homogenized in ice-cold potassium phosphate buffer (50 mmol/L K_2HPO_4, pH 6.0) containing hexadecyltrimethylammonium bromide (HETAB, 0.5%). The homogenate was centrifuged at 15,000*g* for 10 minutes at 4°C, and the supernatant was discarded. The pellet was then rehomogenized with an equivalent volume of 50 mmol/L K_2HPO_4 containing 0.5% HETAB and 10 mmol/L ethylenediaminetetraacetic acid (Sigma, St. Louis, Mo). Tissue MPO activity was assessed by measuring the hydrogen peroxide-dependent oxidation of *o*-dianisidine.2HCl. One unit of enzyme activity is defined as the amount of MPO present that causes a change in absorbance of 1.0 unit/min at 655 nm and 37°C, and expressed in units per gram of tissue.[29]

Measurement of glutathione. Glutathione is a key antioxidant and is used as an indicator of the reduction capacity of the tissue. Glutathione was determined by the spectrophotometric method, which was based on the use of Ellman's reagent.[30,31] Results were expressed in micromoles of glutathione per gram tissue.

Statistical analysis. All statistical analyses were performed with an IBM-compatible (IBM, New York, NY) personal computer using the SPSS 12.0 software (SPSS Inc, Chicago, Ill). Data were expressed as median and quartile. Continuous variables among groups were compared with the Kruskal-Wallis one-way analysis of variance, and statistically significant data were further analyzed with the Mann-Whitney *U* test. A $P < .05$ was regarded as statistically significant.

RESULTS

The STZ-injected rats remained diabetic for 8 weeks before statin treatment. During this period, polydipsia and polyuria were observed in all diabetic rats. Three rats (1 in the diabetic and 2 in the statin pretreated diabetic group) died during the reperfusion phase.

Results of biochemical measurements including blood glucose and lipids are presented in Table I. There was a prominent difference between diabetic and nondiabetic groups in terms of blood glucose and HbA_{1c} levels. However, statin pretreatment did not cause any changes in blood glucose and HbA_{1c} levels. HbA_{1c} levels were found to be significantly higher in the diabetic and statin-pretreated diabetic groups compared with nondiabetic statin-treated and untreated groups ($P < .001$).

No significant difference was observed in the serum cholesterol and HDL levels among groups. Triglyceride and VLDL levels were higher in the diabetic group (81 mg/dL [48 to 96] and 18.4 mg/dL [10.2 to 23.3]) than in the nondiabetic group (34 mg/dL [30 to 48] $P < 0.001$ and 6.8 mg/dL [6.1 to 9.6], $P < .001$), respectively. In diabetic groups, statin pretreatment statistically diminished the creatinine kinase levels, a measure of muscle injury. Creatinine kinase levels were also found to be significantly higher in the diabetic group compared with the nondiabetic group ($P < .001$).

Tissue edema. The diabetic group developed larger water content of the tibialis anterior muscles compared with the nondiabetic group ($P < .05$). Statin pretreatment significantly decreased the water content of the tibialis anterior muscles, both in the nondiabetic ($P < .01$) and the diabetic groups ($P < .01$, Fig 2).

Muscle necrosis. Larger muscle necrosis developed in the diabetic group compared with the nondiabetic group ($P < .05$). The extent of muscle necrosis in the statin pretreated diabetic group was significantly lower than that of the diabetic group ($P < .001$). Despite the presence of a similar trend in nondiabetic groups, it did not reach a statistically significant value (Fig 2, Table II).

Malondialdehyde levels. After lower limb ischemia–reperfusion, MDA levels, as the index of lipid peroxidation, were higher in diabetic group compared with the nondiabetic group, but it did not reach a statistically significant value. Furthermore, statin pretreatment did not reduce the MDA levels in either the diabetic or the nondiabetic group (Fig 3, Table II).

Glutathione levels. After lower limb ischemia–reperfusion in the diabetic group, the glutathione activity, an index of antioxidant capacity, was lower than that in the nondiabetic group ($P < .05$). Six weeks of statin treatment

Table I. Biochemical indicators of the experimental groups

	Nondiabetic	*Statin pretreated nondiabetic*	*Diabetic*	*Statin pretreated diabetic*
Blood glucose (mg/dL)	107 (104-111)*	91 (85-100)*	386 (280-510)	180 (144-464)
HbA_{1c} (%)	1.7 (1.6-1.8)*	1.7 (1.5-1.8)*	2.5 (2.0-2.8)	2.3 (2.0-2.9)
Cholesterol level (mg/dL)	64 (53-72)	56 (47-64)	61 (58-67)	52 (34-58)
Triglyceride (mg/dL)	34 (30-48)	22 (19-57)	81 (48-96)†	36 (20-54)‡
HDL (mg/dL)	31 (25-39)	32 (26-32)	36 (29-37)	31 (24-39)
VLDL level (mg/dL)	6.8 (6.1-9.6)	6.0 (3.8-10.0)	18.4 (10.2-23.3)†	7.3 (4.0-10.9)‡
CK level (IU/L)	585 (350-1652)	461 (460-462)	2476 (1954-3108)§	772 (419-976)¶

HB, Hemoglobin; *HDL,* high-density lipoprotein; *VLDL,* very-low-density lipoprotein; *CK,* creatinine phosphokinase.
Values are median (quartile).
*$P < .001$ compared with the diabetic groups.
†$P < .01$ compared with the nondiabetic group.
‡$P < .05$ compared with the diabetic group.
§$P < .05$ compared with the nondiabetic group.
¶$P < .01$ compared with the diabetic group.

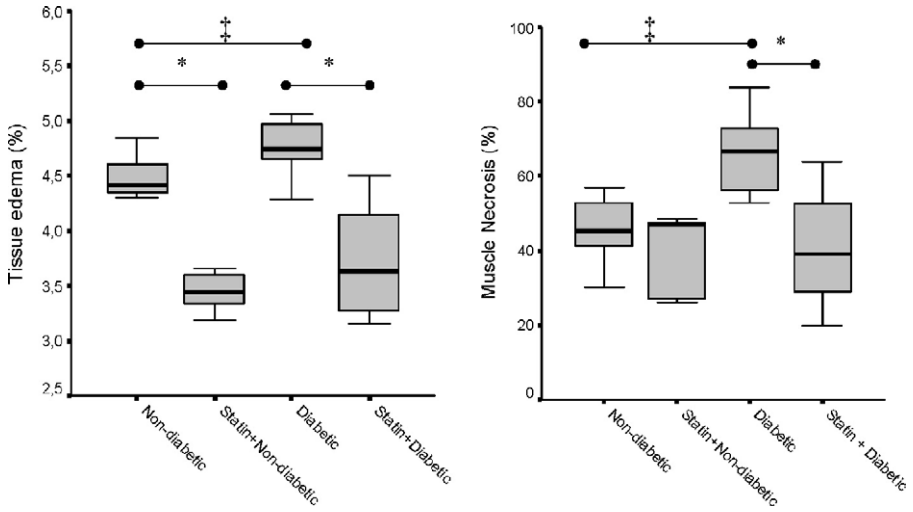

Fig 2. Box plots show the extent of skeletal muscle edema and necrosis determined by the ratio of the wet to dry tissue weight of the gastrocnemius sections. Whiskers show quartile ranges. *$P < .05$, ‡$P < .01$, and †$P < .001$.

Table II. Effects on muscle tissue in the experimental groups

	Nondiabetic	*Statin pretreated nondiabetic*	*Diabetic*	*Statin pretreated diabetic*
Tissue edema (%)	4.4 (4.3-4.6)	3.4 (3.3-3.5)*	4.7 (4.6-5.0)†	3.6 (3.2-4.2)*
Muscle necrosis (%)	45 (38-53)	46 (26-47)	66 (53-75)†	39 (29-53)‡
MDA (nmol/g)	31 (26-33)	33 (28-35)	34 (32-39)	38 (31-44)
GSH (mM/g)	0.28 (0.25-0.34)	0.40 (0.38-0.50)*	0.24 (0.11-0.26)†	0.41 (0.25-0.69)*
MPO (U/g)	28 (26-34)	14 (14-20)*	41 (43-50)†	10 (5-19)*

MDA, Malondialdehyde; *GSH,* glutathione; *MPO,* myeloperoxidase.
Values are median, (quartile).
*$P < .01$ compared with the untreated groups.
†$P < .05$ compared with the nondiabetic group.
‡$P < .001$ compared with the diabetic group.

before induction of ischemia increased the glutathione levels both in the nondiabetic ($P < .01$) and the diabetic groups ($P < .01$; Fig 3, Table II).

Myeloperoxidase levels. After lower limb ischemia–reperfusion in the diabetic group, MPO activity, used as an index of leukocyte accumulation, was higher than that in the nondiabetic group ($P < .05$). Statin pretreatment reduced the MPO activity in the diabetic group compared with the untreated diabetic group ($P < .01$). Similarly, in the nondiabetic group, statin pretreatment significantly

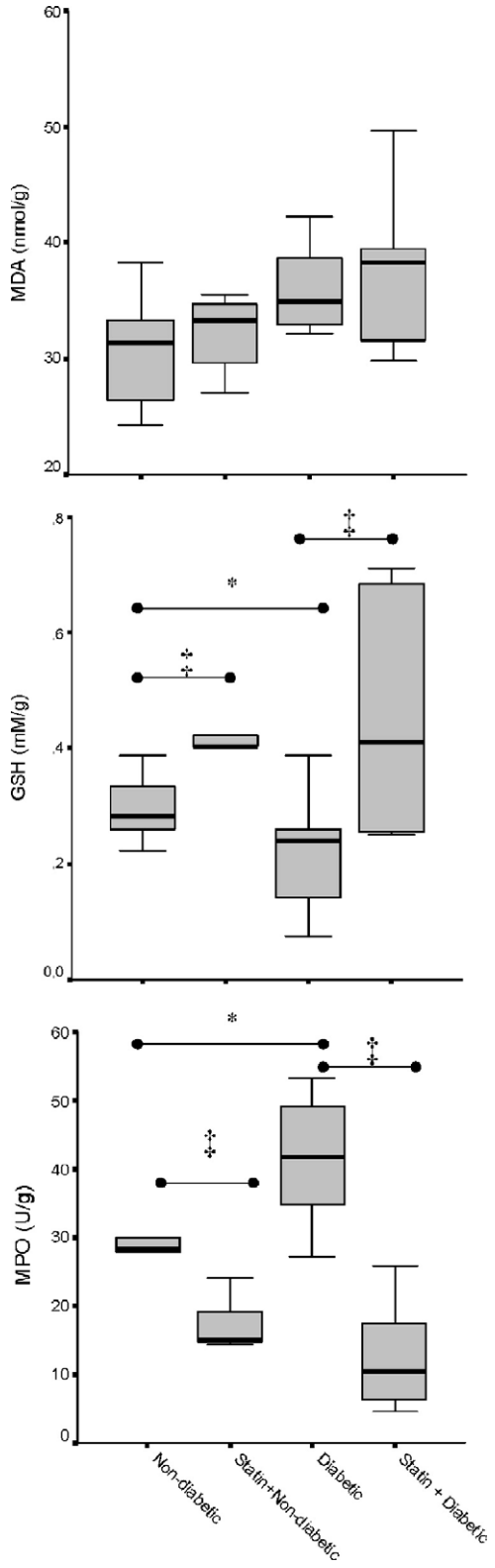

Fig 3. Box plots show the levels of malondialdehyde *(MDA)*, glutathione *(GSH)*, and myeloperoxidase (MPO) in the experimental groups. Whiskers show quartile ranges. $*P < .05$, $‡P < .01$.

reduced the MPO activity compared with the untreated nondiabetic group ($P < .01$; Fig 3, Table II).

DISCUSSION

In this study, we obtained results supportive of the fact that pretreatment with simvastatin for 6 weeks ameliorated the tourniquet-induced skeletal muscle ischemia–reperfusion injury in both diabetic and nondiabetic rats. Statin pretreatment was associated with reduced creatinine kinase levels, tissue edema, muscle necrosis ratio, and MPO activity, as well as increased glutathione levels in diabetic rats subjected to limb ischemia-reperfusion. In nondiabetic rats, statin pretreatment resulted in reduced creatinine kinase levels, tissue edema, MPO activity and increased glutathione levels after limb ischemia–reperfusion. Although many experimental and clinical studies present positive effects of statins on ischemia–reperfusion injury, to our knowledge, this is the first study showing the effects of prophylactic use of statins on acute lower extremity ischemia in an experimental DM model. Results of this study also suggest that DM aggravates the ischemic damage caused by skeletal muscle ischemia–reperfusion injury. In diabetic rats, creatinine kinase levels, tissue edema, muscle necrosis ratio and MPO activities were all increased, and glutathione levels were decreased.

Current evidence suggests that statins may attenuate oxidative stress, inflammation, and platelet aggregation, as well as coagulation, fibrinolysis, and endothelial functions and help to prevent thrombosis or restenosis.[31] Statins may affect the intracellular prenylation of proteins, which modulate the activity of small guanosine triphosphate-binding proteins. This may be an underlying mechanism for some pleiotropic effects of statins.[32]

The protective effects of statins have been suggested in several experimental ischemia models in different tissues:

- Lefer et al[33] showed that simvastatin therapy preserved cardiac contractile functions and perfusion in a rat cardiac ischemia–reperfusion model by reducing the leukocyte-endothelial cell interactions independently from its lipid-lowering effects.
- Amin-Hanjani et al[17] showed that long-term prophylactic treatment with mevastatin upregulated the endothelial nitric oxide synthase and showed neuroprotective effects by augmenting the cerebral blood flow, without altering the serum cholesterol levels.
- Yamada et al[34] pointed out that pretreatment with simvastatin enhanced the blood flow within ischemic brain tissue after middle cerebral artery occlusion.
- Greisenegger et al[35] reported that pretreatment with statins was associated with decreased clinical severity in patients with acute ischemic cerebrovascular events, particularly in patients with diabetes.
- Naidu et al[36] showed that pretreatment with simvastatin was protective against lung reperfusion injury in a rodent model and suggested that the likely mechanisms of protection included altered free radical generation through effects on nicotinamide adenine dinucleotide phosphate

oxidase and preservation of endogenous nitric oxide activity.

- Dillon et al[37] recently demonstrated that pretreatment with simvastatin reduces the tissue oxidative damage and edema associated with skeletal muscle reperfusion injury.

In addition to experimental evidence, findings from prospective and observational clinical studies have demonstrated that statin treatment significantly improves the clinical outcome after cardiovascular events. Data from the Scandinavian Simvastatin Survival Study on 4444 patients with known cardiovascular disease revealed that the use of simvastatin reduced the episodes of new or worsening intermittent claudication by 38%.[38] Treatment with simvastatin in hypercholesterolemic patients with PAD resulted in an increase in pain-free and total walking distances, as well as improvement in physical abilities.[20,39] Simvastatin has been reported to significantly reduce the incidence of cardiovascular events.[12] The recent Diabetes Control and Complications Trial demonstrated that almost all diabetic patients with vascular disease should be taking a statin agent, given the consensus level 1 evidence guidelines.[40]

The present study shows that statins ameliorate tourniquet-induced muscle ischemia–reperfusion injury in the experimental setting. Our results suggest that pretreatment with statins may be useful in preventing and reducing the severity of acute vascular ischemic events. These effects may be most prominent in patients who have circulation challenges such as those seen in diabetic patients. Nevertheless, this implication requires clinical evidence.

The effects of statin pretreatment cannot be accounted for by the confounding systemic variables, because there were no differences between blood glucose, cholesterol levels, and body weights of animals in the treatment group compared with those of untreated controls. A potential mechanism by which statins may exert their beneficial effects is through their direct antioxidant and anti-inflammatory properties.[32,33,36] In this study, we investigated the antioxidant capacity by measuring glutathione levels and observed an increase in glutathione levels with simvastatin therapy. Although we were unable to show reduced MDA levels with statin pretreatment, some of the ability of simvastatin therapy to reduce ischemia–reperfusion injury may be related to its antioxidant effects.

One potential mechanism of action is the ability of statins regulate endothelial nitric oxide synthase.[19] It has been shown recently that pretreatment with statins inhibits leukocyte-endothelial interaction and prevents neuronal death in the rat retina after ischemia-reperfusion injury.[41] Statins have also been shown to downregulate the expression of nuclear factor-κB and activator protein-1 at the transcriptional level, both of which are known to have central roles in ischemia–reperfusion injury.[42] Further studies are required to elucidate the possible mechanisms by which statin pretreatment attenuates skeletal muscle ischemia–reperfusion injury.

Pretreatment with simvastatin had several beneficial effects, including reduced tissue edema, leukosequestration, and increased antioxidant capacity in the nondiabetic group. The reduction of muscle necrosis was not statistically significant, however. The reduction in the extent of necrosis in the simvastatin-treated diabetic group was one that restored it to the level of nondiabetic animals. One possible reason for the lack of improvement in necrosis of the simvastatin-treated nondiabetic animals might be the difference in the basal endothelial nitric oxide synthase (eNOS) levels of diabetic and control animals. Although we did not measure eNOS levels in our study, studies published by Lefer et al[43] show that basal eNOS levels of diabetic hearts are much lower than that of controls, and simvastatin treatment restores the eNOS production and attenuates the extent of necrosis after ischemia and reperfusion of diabetic hearts in vivo. If simvastatin is indeed restoring eNOS expression as suggested by Lefer et al, this could be one way of explaining the mechanism of our findings.

CONCLUSION

In our experimental setting, diabetes mellitus aggravated the ischemic damage after skeletal muscle ischemia–reperfusion injury, and a 6-week therapy with simvastatin reduced the lower limb ischemia–reperfusion injury both in control and diabetic animals. Statin effects were especially dramatic and independent from serum cholesterol levels in diabetic rats. Further clinical trials are necessary to elucidate the effects of statins in acute lower extremity ischemia–reperfusion injury.

AUTHOR CONTRIBUTIONS

Conception and design: CK, DD
Analysis and interpretation: CK, EO, UY, AOH, AK, ED, DD
Data collection: EO, UY, AOH, AK, ED, DD
Writing the article: CK, AC, AK
Critical revision of the article: CK, AC, AK
Final approval of the article: CK
Statistical analysis: CK, EO, AC
Obtained funding: CK
Overall responsibility: CK

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